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Microwave-assisted extraction of bioactive saponins from chickpea (*Cicer arietinum* L)

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Abstract: Growing interest in plant secondary metabolites has brought with it the need for economical, rapid and efficient extraction protocols. Microwave-assisted extraction (MAE) was used to extract saponins from chickpea (*Cicer arietinum*). Several MAE conditions were tested, and the method proved to be superior to Soxhlet extraction with regard to amounts of solvents required, time and energy expended. The use of a butanol/H_2O mixture showed selectivity towards saponin extraction. Using TLC, two distinct saponins were observed in the various chickpea extracts. The identification of the major saponin as a DDMP-conjugated saponin was verified using ^{1}H and ^{13}C NMR, for the first time in chickpea. The MAE procedure most likely contributed to the conservation of the heat-sensitive DDMP moiety. The pure chickpea saponin exhibited significant inhibitory activity against *Penicillium digitatum* and additional filamentous fungi. Two *Fusarium* strains tested were highly tolerant to the saponin. The potential for using MAE for the efficient extraction of natural products may assist in expediting the chemical analysis and characterization of the biological activities of such compounds.
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Keywords: microwave-assisted extraction (MAE); saponins; chickpea; antifungal

INTRODUCTION

The increasing interest in plant secondary metabolites is accompanied by a need to expand and modify the arsenal of plant-extraction protocols. Conventional methods for the extraction of natural products from plant material, eg Soxhlet, liquid–liquid, and solid–liquid extractions are characterized by the consumption of large volumes of solvent and energy, lengthy extraction procedures, and the potentially deleterious degradation of labile compounds. In recent years, new extraction techniques have been developed to reduce the volume of solvent needed for extraction (or to eliminate its use entirely), to reduce extraction and extract clean-up times, and to improve the reproducibility of compound recovery. These recent extraction techniques include accelerated solvent extraction (ASE), supercritical-fluid extraction (SFE), solid-phase microextraction (SPME), extraction with supercritical or subcritical water, and microwave-assisted extraction (MAE).[1–6] Most of these methods have similar pros and cons with regard to solvent volume, extraction time and extraction efficiency. The use of SFE or ASE, however, requires greater financial investment, and the presence of water in samples can cause blockages in both techniques.

The technology for microwave-assisted chemistry has matured significantly since the pioneering work of Ganzler *et al* on the use of microwave energy to accelerate solvent extraction procedures for analytical sample preparation.[7] Demand for increased safety levels and more advanced method development has spurred the growth of microwave-assisted chemistry. As an outcome, internal and peripheral devices have been developed that allow the continuous monitoring and control of internal temperature in individual vessels. This recent availability of commercial microwave equipment that complies with the higher security standards and incorporates closed vessels in their protocols has enabled the extraction of organic pollutants at high pressure and temperature, facilitating rapid and selective analyte desorption from complex matrices—all at a relatively moderate cost. The use of microwave energy enables fast dissolution, drying, acidic digestion and extraction of organic compounds from complex environmental matrices; its main advantages are reduced solvent volume and time consumption, and increased sample throughput.[8,9]

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Although most investigations have been devoted to determining the presence of organic contaminants such as polyaromatic hydrocarbons[10] in environmental samples, the use of MAE has expanded into the pharmaceutical industry.[11,12]

Saponins, which are constitutively produced in many plant species, both in the wild and in domesticated crops, exert a wide range of biological activities. Some saponins have been shown to exhibit antibacterial, antifungal and anti-insect activities and, as such, they have been suggested to constitute part of some plant defense systems. In cultivated crops, including many legumes such as soy, bean, pea and lucerne, triterpenoid saponins are generally predominant. Steroid saponins are common in plants used as herbs or for their health-promoting properties.[13,14] The unique chemical nature of saponins demands tedious and sophisticated techniques for their isolation, structure elucidation and analysis.[15,16] The task of isolating saponins from plant material is complicated even more by the occurrence of many closely related substances in plant tissues, and by the fact that most saponins lack a chromophore. Thus, for many years, the complete characterization of saponins from even well-known saponin-containing plants was limited. However, recent renewed interest in medicinal plants and foods, along with the dramatic evolution of analytical tools, has resulted in a burst of publications presenting numerous novel saponins. The modern methods available for the separation and analysis of saponins have been well reviewed.[16–20]

Chickpea (*Cicer arietinum*), one of the founder crops of the Neolithic agricultural revolution,[21] is the second most important legume for human consumption. It ranks first among Near-Eastern legumes, and is a major source of high-quality dietary protein in the Middle East and on the Indian subcontinent.[22,23] *Fusarium oxysporum* f sp *ciceri* is the causal agent of fusarium wilt of chickpea and inflicts major economic damage to chickpea world-wide.[24] To date, there have been no reports on the isolation and analysis of antifungal activity of any *Cicer*-derived saponin. A single report postulated that *C arietinum* contains only one major saponin, belonging to the soyasaponin group B, which is characterized by a reducing sugar 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) moiety on C-22.[25] The DDMP is a heat-sensitive residue that provides the saponin unique characteristics, including antioxidant capacity, characteristic absorption spectrum and sweet taste.[26]

The objectives of this study were to develop a microwave-based method for the extraction of saponins from chickpea, to establish the presence and stability of DDMP-saponin(s) in chickpea, and to determine whether the MAE extract exhibits antifungal activity.

MATERIALS AND METHODS

Chemicals and media

Solvents of HPLC grade were purchased from JT Baker (Phillipsburg, NJ, USA). Chickpea seeds of the Israeli cultivar Hadas were kindly supplied by Dr S Abbo (The Hebrew University of Jerusalem). Other chemicals were purchased from Sigma (St Louis, MO, USA). Potato dextrose agar (PDA) was purchased from Difco Laboratories (Detroit, MI, USA). Vogel's sucrose (VgS) medium was prepared as described previously.[27]

Extraction procedures

Seed powder

Prior to all extractions, chickpea seeds were ground in a Wiley mill (Arthur H Thomas, Philadelphia, PA, USA) to pass a 2-mm pore-size screen, and dried at 55 °C for 72 h. The dried powder was then extracted using a Soxhlet apparatus with hexane for 6 h to remove all fats.

Microwave-assisted extraction

Defatted powder (4 g) was mixed with a solvent of choice (MeOH, EtOH or EtOH:H_2O 7:3, butanol or butanol:water 1:1; 16 ml) in 20-ml closed vials, which were placed in a mechanically modified microwave oven (ETHOS 1600, Milestone, Sorisole, Italy) and irradiated at 2450 Mhz for 10 or 20 min. The solvent temperature was kept constant at 60 °C using an automatic temperature control device (ATC-FO, Milestone, Sorisole, Italy) submerged into a solvent-containing vessel. Twelve sample TFM (a thermally resistant form of Teflon) vessels were used at a time, with pressure and temperature monitoring capabilities, in a MPR-600/12S rotor (Milestone, Sorisole, Italy). The microwave power was limited to 300 W. After cooling to room temperature, the extract was collected and kept at −20 °C until analysis. Statistical comparisons were made by Student's *t*-test; $p < 0.01$ was considered significant.

Soxhlet extraction

Defatted powder (10 g) was extracted with the solvent of choice (150 ml), for 3 h. After cooling to room temperature, the extract was collected and kept at −20 °C until analysis.

Purification of saponins

The filtrate was loaded onto a C-18 preparative column (C18 Extract-Clean™, Alltech Associates Inc, Deerfield, IL, USA), and impurities were eluted with 600 ml l^{-1} methanol in water. The saponin-containing fraction was eluted with methanol. The eluted fraction was diluted with water (final methanol concentration of 400 ml l^{-1}) and was further purified using HPLC to isolate DDMP-saponins. The HPLC system (Thermo Separation Products, Riviera Beach, FL, USA) was equipped with a diode-array detector (UV6000) and a column oven (35 °C). A reverse-phase C-18 column (250 × 4.6 mm, 'Luna-2' Phenomenex,

Torrance, CA, USA) was employed. Elution was performed using water and methanol, acidified with 0.1 g l^{-1} formic acid, at a flow rate of 1 ml min^{-1}. A chromatography program was developed, starting with an isocratic step at 100 ml l^{-1} methanol for 3 min, then a linear gradient up to 950 ml l^{-1} methanol in 8 min, an isocratic step at 950 ml l^{-1} methanol for 4 min, and equilibrating at the starting conditions for an additional 4 min. The solution was then freeze-dried (HetoDryWinner, Heto-Holten, Gydevank, Denmark) and the saponin powder was kept desiccated at −20 °C. Saponin was quantified using a colorimetric reaction mixture: dry powder (0.2–1 mg) was dissolved in acetic acid (1.5 ml), to which sulfuric acid (1 ml) was added. The absorbance (530 nm) of the reaction product was determined after 15 min incubation at room temperature.[28]

Thin-layer chromatography

Thin-layer chromatography (TLC) was performed using silica gel 60G 20 × 20 cm plates, layer thickness 250 μm (Merck KgaA, Darmstadt, Germany). The solvent system used was butanol:water:acetic acid, 12:2:1 v/v/v (lower phase). Plates were sprayed with *p*-anisaldehyde:acetic acid:sulfuric acid (1:2:100 v/v/v) and heated for 10 min at 110 °C to visualize saponins, or with sulfuric acid in ethanol (10:90 v/v) and heated as above to visualize all substances.[29]

Spectral analyses

The ^{1}H and ^{13}C NMR spectra were recorded using a Bruker (Ettlingen, Germany) 'Avance' DRX-400 instrument, operating at a frequency of 400.13 MHz for ^{1}H observation. The spectrometer was equipped with a 5-mm Bruker inverse multinuclear resonance probe with a single-axis (*z*) gradient coil. Spectra were measured at room temperature in CD_3OD. Chemical shifts (ppm) were given on the δ scale; ^{1}H NMR spectra were referenced to internal tetramethylsilane and ^{13}C NMR spectra to the solvent.

Antioxidant capacity

The total antioxidant activity of the DDMP-saponin was measured by the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay, involving preformed $ABTS^{\bullet+}$ radical cation.[30] Aliquots (100 μl) of increasing concentrations of DDMP-saponin dissolved in 500 ml l^{-1} MeOH in water were added to a 1 ml working solution of $ABTS^{\cdot+}$, vortex mixed and the absorbance (at 734 nm) was measured after 10 min. Appropriate solvent blanks were assayed and the absorbance values of the samples at each time point were subtracted from the blank. All assays were repeated at least three times.

Cultures and growth conditions

Fungal species used in this study were: *Neurospora crassa* (74-OR23-1A), *Fusarium oxysporum* f sp *melonis*, *Fusarium oxysporum* f sp *ciceri*, *Pleurotus ostreatus* Florida F-6, *Sclerotium rolfsii*, *Sclerotinia sclerotiorum* and *Penicillium digitatum*. *Pythium aphanidermatum*, an Oomycete, was also used to assess the activity of the chickpea saponin.

All the fungi (excluding *N crassa*) and *P aphanidermatum* were cultured on PDA. *N crassa* was cultured on VgS medium. *S sclerotiorum* and *P digitatum* were cultured at 25 °C, *Fusarium* isolates were cultured at 27 °C, *P ostreatus*, *S rolfsii* and *P aphanidermatum* were cultured at 30 °C, and *N crassa* was cultured at 34 °C.

DDMP-saponin was prepared as described and dissolved in H_2O:MeOH (3:1 v/v) to a final concentration of 25 mg l^{-1}. The dissolved saponin was then added to the warm medium (50 °C), to a final concentration of 0.25 mg ml^{-1}, and 5-ml aliquots were poured into 50-mm diameter Petri dishes and allowed to solidify. H_2O:MeOH (3:1 v/v) (40 μl) was added, as a control, to otherwise nonamended media. A disk containing growing hyphae of the test organism was placed at the center of the dish and radial growth was measured when the hyphae of the control culture reached 4/5 of the dish diameter.

RESULTS AND DISCUSSION

Several conditions were tested to determine the efficiency of saponin extraction from chickpea defatted powder. TLC profiles of extracts obtained by MAE using several alternative solvents for extraction were qualitatively compared (Fig 1). The most efficient extraction appeared to be 20 min with 700 ml l^{-1} ethanol, as visualized using sulfuric acid staining (lane 2). Extending the extraction time to 40 min in the same solvent (700 ml l^{-1} ethanol) did not result in any observable effect. Moreover, the profiles of extracts obtained with 700 ml l^{-1} ethanol for 20 min with the MAE process and extracts after 3 h of the Soxhlet process showed similar band intensities, suggesting that maximal yield could be obtained with 20 min of MAE.

The heat production process may be the most important variable in a successful microwave assisted extraction of temperature labile compounds. Thus the maximum power of the oven was limited (using a computer controller) to 300 W. To fully control the process we also limited the actual temperature (Fig 1) within the extraction vessels. We used segmented rotors that allow for an increased number of vessels, each of which can be individually inserted and removed. Each vessel segment is sequentially numbered for position indexing and automation. Segmented rotors also come with a central 'chimney' manifold which distributes cooling air to every vessel. Forced air flow is directed on the vessel shields at the liquid level line to dissipate heat.

We tested the possibility of using a mixture of butanol and water, solvents that do not mix, with the microwave apparatus (lane 5). While not many substances were extracted by butanol alone (lane 4), using the butanol:water mixture (1:1 v/v) in the extraction vessel resulted in a profile similar to

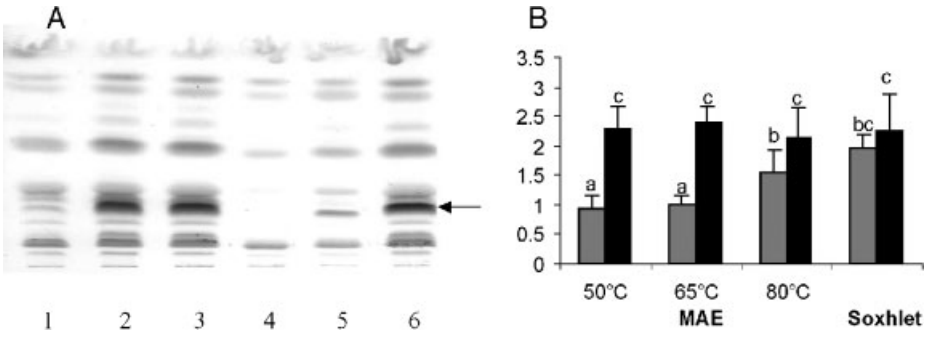

Figure 1. Recovery of DDMP-saponin from ground chickpea using microwave-assisted extraction (MAE) with different solvents and temperatures. A: TLC of chickpea extracts: MAE-methanol, 20 min (1); MAE-700 ml l^{-1} ethanol, 20 min (2); MAE-700 ml l^{-1} ethanol, 40 min (3); MAE-butanol, 20 min (4); MAE-butanol:water (1:1), 20 min (5); and Soxhlet-700 ml l^{-1} ethanol, 3 h (6). MW power, 1500 W; 50 °C, 4 ml solvent g^{-1} dry matter. An extract equivalent to 20 mg of defatted powder was applied to each lane. The plate was stained with 100 g kg^{-1} sulfuric acid in ethanol and heated for 10 min at 110 °C. Arrow indicates major saponin band. B: Saponin (g per 100 g seed dry weight) recovered from three serial extractions at 5-min intervals, each, pooled together. Solvents used were methanol (black) or 700 ml l^{-1} ethanol in water (gray), each at the indicated temperatures. Saponin levels extracted by Soxhlet (6 h) are presented for comparison. Bars represent means ± standard deviation ($n = 5$); different letters represent statistical significance level of $p \leq 0.01$.

that observed using the ethanol:water solution. One explanation for this result may be that, within the pressurized vessel, and in the presence of saponins that may act as emulsifiers,[31] a solvent emulsion was formed. However, comparing lanes 4 and 5 also demonstrates the selectivity of the extraction process.

A major saponin band (indicated by the arrow in Fig 1A) was observed at $R_f = 0.25$. The identity of this band as a saponin was primarily confirmed by anisaldehyde staining showing a violet-blue band at the same R_f (data not shown). This major saponin was clearly observed in the butanol:water extract (lane 5) but could not be detected in the butanol-only extract (lane 4). Contrary to a previous report mentioning the presence of only one saponin in chickpea,[25] a second violet-blue band was observed at $R_f = 0.5$ (lanes 1, 2, 3, 5, and 6), suggesting the presence of other saponins in chickpea seeds. Staining the plates with anisaldehyde reagent, while being informative with regard to the nature of the observed substances, is of limited quantitative value due to poor color stability.

We used direct densitometry to evaluate the amounts of saponins in the various extracts.[32] A linear correlation was observed when increased amounts of purified saponin were applied to the TLC plate, and stained with sulfuric acid (data not shown). The amounts of the major saponin were in the same range in the more efficient extractions (lanes 2, 3 and 6).

The conditions for optimal recovery of saponins from defatted chickpea seeds were evaluated using methanol or ethanol:water (7:3 v/v) and pooling extracts from three serial extractions performed at 5-min intervals. The combined and dried extracts were partitioned between water and butanol, and the dry weight of the butanol-solubles was determined as saponin dry weight (Fig 1B). The levels of saponins in chickpea were found to account for 25 mg g^{-1} of the seed dry weight. On the basis of our observations, the methanol extraction was superior to the ethanol:water extraction at all temperatures. The amounts of saponins extracted by methanol did not change with temperature whereas their amounts in the ethanol:water extract increased with increasing temperature. The amounts extracted by three intervals of ethanol:water at 80 °C were similar to those extracted at 6 h by Soxhlet.

Microwave-assisted extraction gains increasing interest as an advantageous method for the extraction of natural products, since its first patenting in the early 1990's.[6,8] Purification of saponins traditionally consists of a liquid–liquid extraction step following the extraction procedure. The amphipathic nature of these compounds makes this extraction tedious and inefficient. In this work, butanol and water, that form separate phases (layers), were used in a single extraction step. Including the liquid–liquid extraction in the MAE process thus enhanced the efficiency and selectivity of the MAE process. The choice of solvents used in the MAE may serve as a separation tool.

The methanol-MAE extract was used for further purification and identification of the major saponin in chickpea. The saponin was resolved using a preparative C-18 column and then an analytical C-18 column (Fig 2). A substance exhibiting λ_{max} at 292 nm (Fig 2, inset), which is characteristic of the DDMP moiety in soy saponins, eluted at $t_R = 16.4$ min. This

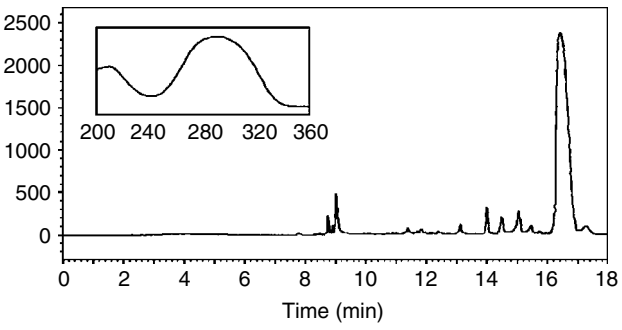

Figure 2. Separation and identification of DDMP-saponin from chickpea. RP-18 chromatogram monitored at 280 nm. A substance with absorbance maximum at 292 (inset) was eluted at 16.4 min.

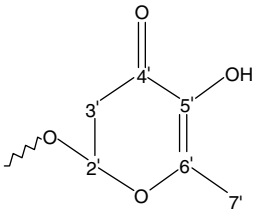

Figure 3. Schematic structure of a DDMP moiety.

unique monosaccharide chromophore (Fig 3) allows the identification and quantitative determination of saponins.

The substance was collected and analyzed by ^{1}H and ^{13}C NMR (Table 1). ^{1}H NMR and ^{13}C NMR spectra identical to the data reported for a DDMP moiety by Kudou *et al*[26,33] were recorded. These results support the earlier assumption that a DDMP moiety is conjugated to the major saponin in chickpea. The amount of DDMP-saponin was also determined through monitoring the chromophore produced in the reaction of saponins with sulfuric acid in acetic acid (530 nm), and a linear correlation ($R^2 = 0.997$) was observed in the range of 0.2 to 1 mg. We also found a linear correlation ($R^2 = 0.993$) of the reducing activity of the pure saponin towards an oxidized cation radical ($ABTS^{\cdot+}$), in the range of 0–100 μg saponin. It was previously shown, in several other legume seeds, including soy,[26] *Phaseolus coccineus*[34] and *Dolichos lablab*,[35] that a conjugated DDMP moiety will provide the saponin with reducing potential. Here we have, for the first time, demonstrated the presence of a DDMP-conjugated saponin in chickpea.

The antimicrobial activity of some saponins has long been recognized.[36,37] Some reports describe the anti-yeast activity of saponins as having an anti-food-deterioration effect.[38] The fact that chickpea is an edible crop increases the potential attractiveness of the saponin described here as a potential food preservative, due to its 'generally recognized as safe' (GRAS) nature. We assayed the purified saponin for its activity against several fungal species, including plant pathogens, post-harvest pathogens and molds, as well as against an Oomycete phytopathogen. Relative linear growth of filamentous fungi and the Oomycete in the presence of chickpea DDMP-saponin was monitored. Linear growth was determined by measuring the test colony diameter at the time at which the nonamended control cultures covered 4/5 of the Petri dish diameter (Fig 4).

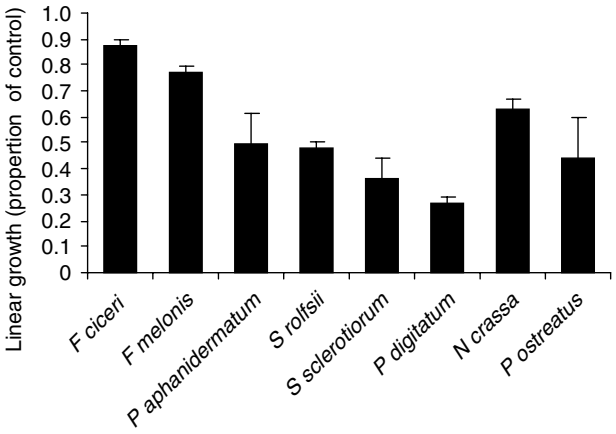

Figure 4. Inhibition of linear growth by the addition of DDMP-saponin, presented as percentage of linear growth of the test microorganism, in control nonamended media, at one selected time point. Values represent means and bars represent standard deviation ($n = 6$).

All the organisms studied were inhibited, to various degrees, by the DDMP-saponin. The extent of the inhibition ranged from 12% in the case of *F oxysporum* f sp *ciceri* to 73% in the case of *P digitatum*. The direct potential role of chickpea saponins in conferring resistance of the plant to a variety of fungi has yet to be determined. Avenacin is an antifungal saponin in oat that has been extensively studied with regard to its biosynthesis and modes of action.[39–44] Its predominant mode of antifungal action has been suggested to involve interference with the sterol component of the fungal plasma membrane.[42] This interaction can lead to the formation of pores in the membrane and, subsequently, to nonspecific leakage and cell death.

There are several ways to explain the differences in fungal growth inhibition by saponins. Among them are differences in sterol (a potential target) composition and saponin detoxification (as was demonstrated in the case of *Gaeumannomyces graminis* var *avena*.[45] It is not surprising that *F oxysporum* f sp *ciceri* was the most resistant among the organisms tested, as it is a natural pathogen of chickpea. It is conceivable that the *F oxysporum* f sp *melonis* strain, even though not a pathogen of chickpea, is sufficiently close, evolutionarily, to *F oxysporum* f sp *ciceri* to be similarly tolerant to the saponin. The other fungi exhibited significantly lower tolerance levels to the saponin.

Table 1. ^{1}H NMR and ^{13}C NMR chemical shifts (s (singlet), d (doublet) and dd (double doublet)) and proton ± proton coupling constants (*J*, Hz) for the saccharide DDMP moiety of chickpea saponin in CD_3OD, compared with literature data (acquired in DMSO-d_6)

^{1}H	Chickpea saponin	Soya saponin β-g (Kudou *et al*[26])	^{13}C	Chickpea saponin	Soya saponin β-g (Kudou *et al*[26])
C-2′	5.26 dd (3.5, 3.5)	5.38 dd (3, 3)	C-2′	98.5	96.6
C-3′a	2.94 dd (13.2,3.9)	2.93 dd (14, 3)	C-4′	187.8	185.2
C-3′b	2.53 dd (13.1,3.6)	2.35 dd (14, 3)	C-5′	134.5	132.9
			C-6′	155.9	152.5
C-7′	1.98 s	1.90 s	C-7′	15.55	15.2

The sensitivity of *Pythium* to the saponin was somewhat unexpected. Sterols, even though shown to constitute powerful signaling components for Pythiaceae, are not necessarily required in the physiology of these Oomycetes.[46] A possible explanation for this observation is that the chickpea saponin can impair *Pythium* growth via an alternative (non-sterol-dependent) mechanism.

In this report, we demonstrated the feasibility and ease of MAE for the isolation of a chickpea saponin. Our results show that saponins constitute 25 mg g^{-1} of the seed dry weight, and that the major portion of these saponins is a DDMP-conjugated saponin with antifungal properties. The potential for using MAE for the efficient extraction of natural products may assist in expediting the chemical analysis and characterization of the biological activities of such compounds.

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